Chymotrypsin Catalysis. Evidence for a New Intermediate[1]

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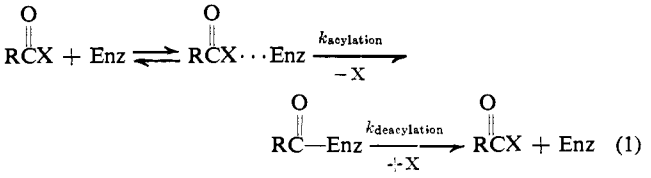
Abstract: Rates of hydrolysis of acetyl-L-tryptophan *p*-methoxy- and *p*-chloroanilides have been measured spectrophotometrically. The principal observations are as follows. (1) V_{max} for the methoxyanilide is approximately three times that for the chloroanilide at pH 8. (2) V_{max} for the chloroanilide is constant, within experimental error, in the pH range 8–5.6, and is larger than the corresponding constant for the methoxyanilide at pH 5.8 and below. (3) K_m for the chloroanilide is about three times smaller than K_m of the methoxyanilide at pH 8 and these constants are approximately equal at pH 5.8. (4) The K_m for the methoxyanilide is doubled as the pH is decreased from pH 8 to 5.8. (5) The pH dependence of V_{max} with the methoxyanilide is consistent with a mechanism in which a single group with a pK of 6.6 is required in the ionized form for activity. The pK for catalytic activity with the chloroanilide is less than 6. A mechanism consistent with these results as well as the positive Hammett ρ observed for the second-order rate constant for acylation of chymotrypsin with substituted phenyl esters[2] involves a scheme in which a tetrahedral intermediate precedes the formation of an acyl enzyme. Formation of the tetrahedral intermediate is rate limiting in reactions of esters and breakdown of the tetrahedral intermediate is rate limiting with anilides. The substituent dependence of K_m and the pK (but not V_{max}) may, alternatively, be accounted for if the pK of the Michaelis complex is influenced by the nature of the anilide substrate.

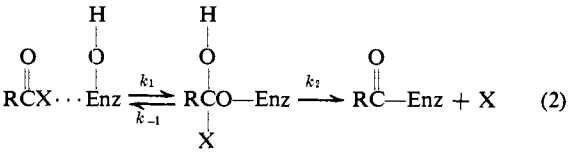
Rates of deacylation of benzoylchymotrypsin intermediates formed in the chymotrypsin-catalyzed hydrolysis of activated benzoyl derivatives are increased by electron-withdrawing *para* and *meta* substituents.[3] These results have been interpreted as suggesting the involvement of some form of base catalysis in the deacylation mechanism. Similar observations have been made in studies of chymotrypsin acylation with substituted phenyl acetates for which the Hammett ρ for the second-order rate constant for acylation ($k_{acylation}/K_m$) is 1.8.[4] If substituent effects on K_m are unimportant the involvement of basic catalysis is, therefore, suggested for the acylation reaction. This is to be expected from the symmetry of the acylation and deacylation processes (see eq 1). It is, therefore, surprising



(1)

that the substituent dependence for the hydrolysis of acylaminotyrosine anilides[5] is the reverse of that observed with esters and the maximum velocities for these reactions are increased by electron-donating substituents. The rate-limiting step in anilide hydrolysis by chymotrypsin is enzyme acylation[6] so that substituent effects on the same reaction are opposite for ester and anilide substrates. The electronic requirements for anilide reactions have been attributed to the involvement of acid catalysis in the acylation reaction since this mode for catalysis will be facilitated by electron donation by the aniline portion of the substrate. The principal objection to this assignment is that the pH dependence of both acylation and deacylation is consistent with a mechanism in which an imidazole side chain of a histidine residue is required in the basic form for activity. There is no evidence implicating the acidic form of any other residue in the bond-making and -breaking steps.

The disparate electronic requirements of anilides and esters suggest that the same step is not rate limiting with these two classes of substrates. For example, in the scheme outlined in eq 2, the rate-limiting step may



(2)

involve either formation or breakdown of a tetrahedral intermediate. The substituent dependence for anilides and esters can be accounted for by a scheme in which tetrahedral intermediate formation is rate limiting with esters and breakdown of the tetrahedral intermediate is rate limiting with anilides. We provide evidence here for breakdown of a tetrahedral intermediate to the acyl enzyme being rate limiting with anilides.

Experimental Section

Materials. Three times crystallized α-chymotrypsin was obtained from Worthington. Acetyl-L-tryptophan, from Mann, was

(1) Supported by Grant No. DE 2761-01 from the National Institute for Dental Research.

(2) M. L. Bender, *J. Am. Chem. Soc.*, **84**, 2577 (1962).

(3) (a) M. Caplow and W. P. Jencks, *Biochemistry*, **1**, 883 (1962); (b) S. A. Bernhard, E. Hershberger, and J. Keizer, *ibid.*, **5**, 4120 (1966).

(4) (a) M. L. Bender, *J. Am. Chem. Soc.*, **84**, 2577 (1962); a Hammett ρ equal to 1.43 based upon two points is reported for V_{max} for acylation of chymotrypsin with phenyl trimethylacetates. In these studies the maximum substrate concentration was approximately 3% of the estimated K_m.

(5) (a) W. F. Sager and P. C. Parks, *ibid.*, **85**, 2678 (1963); (b) W. F. Sager and P. C. Parks, *Proc. Natl. Acad. Sci. U. S.*, **52**, 408 (1964); (c) T. Inagami, S. S. York, and A. Patchornik, *J. Am. Chem. Soc.*, **87**, 126 (1965); (d) H. F. Bundy and C. L. Moore have reported (*Biochemistry*, **5**, 808, 1966) a study of the reactions of the *p*-, *m*-, and *o*-nitroanilides of benzoyltyrosine under conditions where (S) < or $\ll K_m$. The rates for these reactions are increased with increases in electron withdrawal. (e) L. Parker and J. H. Wang, *J. Biol. Chem.*, **243**, 3729 (1968).

(6) (a) T. Inagami and J. M. Sturtevant, *Biochem. Biophys. Res. Commun.*, **14**, 69 (1964). The results described here do not conclusively implicate an acyl-enzyme intermediate in the hydrolysis of anilides since reactions were studied at a single low concentration of the racemic substrate. Therefore, increases in the rate induced by hydroxylamine may be compensated for by an increase in K_m in the presence of hydroxylamine. K_m for acetyltyrosinehydroxamic acid is increased by hydroxylamine (M. Caplow and W. P. Jencks, *J. Biol. Chem.*, **239**, 1640 (1964)). (b) That acyl-enzyme formation is rate limiting may be concluded from the fact that rates for all anilides are not equal.

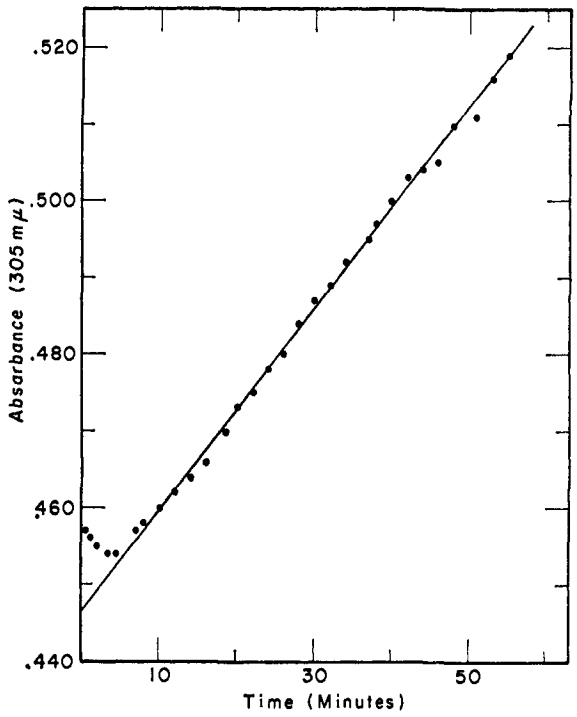


Figure 1. Chymotrypsin hydrolysis of acetyltryptophan *p*-methoxyanilide at pH 5.53. The substrate concentration was 8.0×10^{-4} *M*.

converted to the anilide by the method of Anderson, *et al.*[7] A solution of 1.23 g (5 mmol) of acetyltryptophan and 0.6 ml of N-methylmorpholine in 25 ml of tetrahydrofuran was cooled to 0°. The resultant solution was stirred vigorously as 0.65 ml of isobutyl chloroformate was added, followed 1 min later by 5.5 mmol of the appropriate aniline dissolved in 3.5 ml of tetrahydrofuran. The solution was allowed to come to room temperature and was stirred for 2 hr more. After the solvent was removed under vacuum the resultant solid was dissolved in 40 ml of ethyl acetate and this solution was washed twice, first with 10 ml of 1 *N* HCl and then with saturated sodium bicarbonate. After washing the ethyl acetate several times with water the material was taken to dryness under vacuum. The methoxyanilide was crystallized twice from ethyl acetate to give a white product, mp 197–198°. *Anal.* Calcd for $C_{20}H_{21}N_3O_3$: C, 68.36; H, 6.02; N, 11.96. Found: C, 68.37; H, 6.06; N, 11.71. The chloroanilide was crystallized two times from benzene to give a slightly yellowish material, mp 162–163°. *Anal.* Calcd for $C_{19}H_{18}ClN_3O_2$: C, 64.12; H, 5.10; N, 11.81. Found: C, 64.27; H, 5.17; N, 11.81. Spectro quality dimethyl sulfoxide obtained from Matheson Coleman and Bell was used without further purification.

Methods. Chymotrypsin solutions were made up daily in 10^{-3} *M* HCl and were stored on ice. Reactions were carried out in stoppered cuvettes in a thermostated cell compartment of a Zeiss PMQII or a Cary 15 spectrophotometer. The last component to be added to reaction mixtures was the substrate so as to take advantage of the fact that in the presence of enzyme the anilide substrates form stable supersaturated solutions. The maximum concentrations of the methoxyanilide and chloroanilide used in kinetic experiments were, respectively, approximately 10 and 1.2 times the saturation concentration. The stability of supersaturated solutions is indicated by the good correspondence of extrapolated zero time absorbance measurements to Beer's law (305 mμ), the excellent zero-order plots obtained from initial rate data, the good correspondence of rates with the Michaelis–Menten equation, and the equivalence of rate constants obtained by spectrophotometric and titrimetric assays (see below). Also with both substrates at both the highest and lowest concentrations used (see Table II), at pH's 5.5 and 8.0, the rate of absorbance change was found to be proportional to the enzyme concentration over a two- to fivefold range which spanned the enzyme concentration used in kinetic experiments reported in Table II. The rate of absorbance change extrapolated to zero enzyme concentration is *zero*. With reaction mixtures containing the maximal concentration of the methoxyanilide substrate visual inspection in a darkened room with a spotlight illuminating the cuvette revealed that a small number (about 20) of barely visible crystal nuclei appeared during the time necessary for the optical assays. In the absence of the enzyme crystallization is somewhat erratic and occurs in about 2–4 min resulting in a totally opaque solution. Addition of substrate to buffered solutions without enzyme gave rise to a decreased in absorbance over a period of approximately 5 min after which time no further absorbance changes were observed. The absorbance decrease at 305 mμ was

(7) G. R. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Am. Chem. Soc.*, **89**, 5012 (1967).

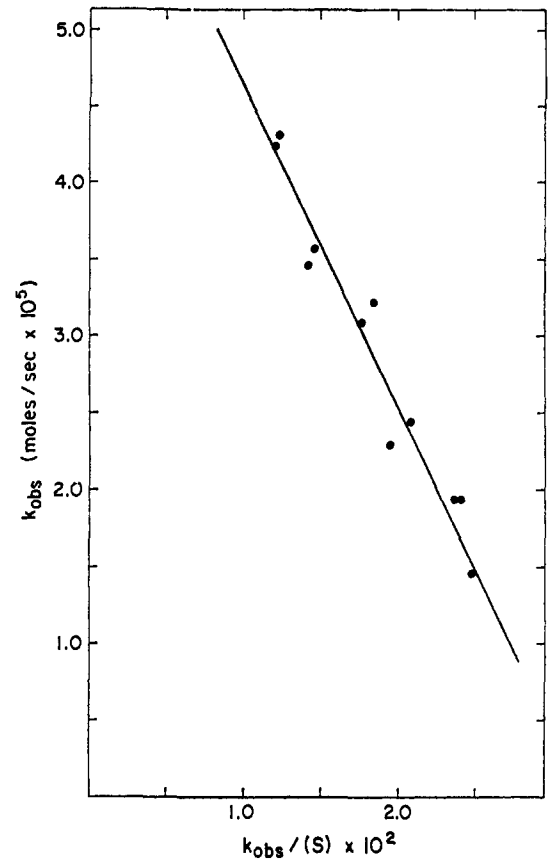


Figure 2. Effect of substrate concentration on the chymotrypsin hydrolysis of acetyltryptophan *p*-methoxyanilide at pH 7.65.

Table I. Extinction Coefficient at 305 mμ for Acetyltryptophan Anilides and Related Compounds[a]

Compound	pH	Molar extinction coefficient, cm^{-1}
Acetyltryptophan *p*-chloroanilide		350
Acetyltryptophan *p*-methoxyanilide		347
Acetyltryptophan	>5.5	430
p-Chloroaniline	5.5–8.1	813
p-Methoxyaniline	5.60	1140
	5.82	1270
	6.30	1590
	7.70	1747
	8.18	1747

[a] Conditions were identical with those for kinetic measurements reported in Table II. The extinction coefficients were not measurably affected by ionic strength variations described in Table II.

Table II. Chymotrypsin Hydrolysis of Acetyltryptophan Anilides at 25° [a]

p-Methoxyanilide						p-Chloroanilide						
pH and buffer [b]	Ionic strength [c]	Substrate concn × 10^3, M	No. of points	V_{max} [d] × 10^2, sec^{-1}	K_m [d] × 10^3, M	pH and buffer [b]	Ionic strength [c]	Substrate concn × 10^3, M	No. of points	V_{max} [d] × 10^2, sec^{-1}	K_m [d] × 10^3, M	$V_{max}^{methoxy}/V_{max}^{chloro}$
8.07 A	0.05	0.65–2.50	10	2.21 ± 0.017	1.97 ± 0.27	8.07 A	0.05	0.30–1.70	12	0.676 ± 0.014	0.628 ± 0.032	3.26
8.08 A	0.05	0.50–2.50	10	2.20 ± 0.27	1.96 ± 0.42	8.10 A	0.05	0.30–1.10	10	0.745 ± 0.045	0.726 ± 0.087	2.95
						8.14 A	0.05	0.40–1.10	10	0.731 ± 0.028	0.609 ± 0.047	
8.14 B	0.16	0.83–3.54	13	2.93 ± 0.15	1.90 ± 0.21	8.18 B	0.16	0.33–1.11	12	0.950 ± 0.072	0.774 ± 0.11	3.08
7.64 C	0.20	0.58–3.50	13	2.82 ± 0.15	2.11 ± 0.11	7.64 C	0.20	0.30–1.10	10	0.809 ± 0.031	0.574 ± 0.047	3.49
7.64 C	0.20	0.59–3.57	12	2.88 ± 0.11	1.91 ± 0.15							
6.28 D	0.06	0.59–3.56	12	0.907 ± 0.10	2.77 ± 0.53	6.30 D	0.06	0.30–1.10	10	0.966 ± 0.11	2.65 ± 0.39	0.94
6.27 E	0.16	0.60–3.62	12	0.897 ± 0.060	2.52 ± 0.31	6.27 E	0.16	0.34–1.14	10	0.795 ± 0.049	1.70 ± 0.16	1.13
5.84 F	0.07	0.56–2.38	9	0.358 ± 0.057	3.38 ± 0.81	5.83 F	0.07	0.40–1.10	10	0.521 ± 0.069	2.73 ± 0.54	0.69
5.81 F	0.20	0.52–2.61	13	0.534 ± 0.065	4.45 ± 0.75	5.82 F	0.20	0.33–1.11	12	0.680 ± 0.172	3.72 ± 1.15	0.78
5.83 F	0.20	0.52–2.58	10	0.527 ± 0.048	4.42 ± 0.56	5.81 F	0.20	0.26–1.11	10	0.870 ± 0.22	5.62 ± 1.62	0.61
5.53 G	0.16	0.32–1.60	12	0.288 ± 0.045	4.85 ± 0.95	5.60 G	0.16	0.30–1.11	10	0.630 ± 0.159	5.33 ± 1.9	0.46

[a] Reactions carried out in 10% dimethyl sulfoxide. The enzyme concentration was 1.5 and 4.0 (±10%) × 10^{-5} M, respectively, for reactions with the chloroanilide and methoxyanilide. [b] The buffer composition is as follows: A, 0.1 M Tris; B, 0.05 M Tris; C, 0.08 M phosphate; D, 0.05 M phosphate; E, 0.08 M imidazole; F, 0.08 M acetate; G, 0.04 M acetate. [c] Ionic strength contribution from buffer ions and added KCl. [d] The standard error is given after the kinetic constants.

0.013 with 1.1 × 10^{-3} M methoxyanilide (pH 5.5), and 0.008 (pH 8.0) and 0.0015 (pH 5.5) with 1.2 × 10^{-3} M chloroanilide.

Rates of anilide hydrolysis were followed from 2.5 to 25% to completion with most reactions involving approximately 10% total consumption of substrate. This corresponds to an absorbance change of approximately 0.05. Twenty to forty absorbance measurements were recorded in each kinetic run and except for results obtained in the initial phase of the reaction few readings deviated from a linear zero-order plot by more than 0.002 optical density unit. The slit width was maintained constant for a set of kinetic runs, and the absorbance of concentration solutions was brought into an easily readable range by modifying the detector attenuation. Infinite time optical density measurements were obtained in single runs with both substrates; with the chloro- and methoxyanilides these values were 101 and 89% of theory, respectively. The accuracy and validity of the spectrophotometric assay were verified by results obtained using a titrimetric assay in a study of the hydrolysis of 1.7 × 10^{-3} M methoxyanilide at pH 7.70, ionic strength 0.2. With the enzyme concentration equal to 4.0 × 10^{-5} M the observed first-order rate constant was 1.23 × 10^{-2} sec^{-1}, which is to be compared with a rate equal to 1.35 × 10^{-2} sec^{-1} calculated from results obtained at pH 7.64 (see Table II) with the spectrophotometric assay. Zero-order rate constants obtained from the slope of a plot of absorbance *vs.* time were converted to first-order rate constants by use of the extinction coefficients given in Table I and a molecular weight for chymotrypsin equal to 25,000. Values of V_{max} and K_m were obtained by a computer fit to the Michaelis–Menten equation as described by Hansen, Ling, and Havir.[8]

Results

The rates of hydrolysis of acetyltryptophan *p*-methoxy- and *p*-chloroanilides have been measured spectrophotometrically. Reactions closely fit a zero-order plot except for reactions of the methoxyanilide at pH 5.8 and 5.5 where a slight lag precedes the zero-order reaction (Figure 1). This lag is associated with some nonenzymatic reaction of the substrate. Reactions exhibit typical saturation kinetics and the results obtained in a representative series of rate measurements are plotted according to eq 3 in Figure 2. Results are summarized in Table II. Although in most cases the enzyme was only fractionally saturated the precision of the results permits considerable confidence in the reported kinetic constants. For example, in the hydrolysis of acetyltryptophan *p*-methoxyanilide at pH 5.8, ionic strength 0.16, the range for V_{max} and K_m calculated from the Students t test is 0.469–0.585 sec^{-1} and 3.79–5.21 × 10^{-3} M, at the 75% confidence level. Approximately similar limits hold for the other constants. The principal observations can be summarized as follows. (1) V_{max} for the methoxyanilide is approximately three times that for the chloroanilide at pH 8. (2) V_{max} for the chloroanilide is constant, within experimental error, in the pH range 8–5.6 and is larger than the corresponding constant for the methoxyanilide at pH 5.8 and below. (3) K_m for the chloroanilide is about three times smaller than K_m for the methoxyanilide at pH 8 and these constants are approximately equal at pH 5.8. (4) The K_m for the methoxyanilide increases by a factor of approximately 2 on going from pH 8 to 5.8.

$$k_{obsd} = V_{max} - K_m(k_{obsd}/S) \quad (3)$$

The pH dependence of V_{max} with the methoxyanilide is consistent with a mechanism in which a single group is required in the ionized form for catalytic activity. Using results obtained at pH 8 (at the appropriate ionic strength) to estimate the catalytic activity of the fully ionized form of the enzyme the calculated pK from results obtained at pH's 6.3 (excepting the reaction in

(8) K. R. Hanson, R. Ling, and E. Havir, *Biochem. Biophys. Res. Commun.*, 29, 194 (1967). We are grateful to Dr. Hanson of the Connecticut Agricultural Experiment Station for providing the computer program.

buffer E), 5.8, and 5.5 is 6.60 ± 0.06. The pK calculated from results obtained in buffer E at pH 6.27 is 6.78. The pH invariance of rates with the chloroanilide indicates that the pK for catalytic activity with this compound is less than 6.

The substituent dependence of V_{max} and K_m with the two acetyltryptophan anilides reported here is surprisingly different from that observed with the corresponding acetyltyrosine compounds.[5e] With the latter substrates at pH 8 V_{max} for the methoxy compound is 11.5 times that of the chloro compound and K_m for the chloro compound is 39 times smaller than the corresponding constant with the methoxy derivative. The ratio V_{max}/K_m for the acetyltyrosine and acetyltryptophan chloro- and methoxyanilides is similar. The reaction conditions for these studies were similar except that in the study reported here the dimethyl sulfoxide concentration was 10% rather than 27%, and 0.25 M calcium chloride was omitted.

Discussion

Results obtained here with acetyltryptophan *p*-methoxy- and *p*-chloroanilide, as well as those obtained previously with other anilides and with esters for which the acylation step has been measured directly, are consistent with the mechanism outlined in eq 2. The rate law for the initial rate of acyl-enzyme formation according to this scheme is

$$\text{rate} = \frac{k_1k_2}{k_{-1} + k_2}(\text{RC}(\text{O})\text{X}\cdots\text{Enz}(\text{OH})) \quad (4)$$

In a two-step reaction of this type the nature of the rate-limiting step is determined by the relative size of k_{-1} and k_2. It is proposed that for phenyl ester substrates $k_2 > k_{-1}$ so that

$$\text{rate} = k_1(\text{RC}(\text{O})\text{X}\cdots\text{Enz}(\text{OH})) \quad (5)$$

and the rate-limiting step involves formation of the tetrahedral intermediate. The positive Hammett ρ observed for reactions of phenyl acetates[4a] is consistent with this scheme since the attack step will be facilitated by electron withdrawal. That $k_2 > k_{-1}$ results from the fact that the k_2 step involves displacement of a more acidic leaving group. In studies of nonenzymatic alcoholysis of esters it has been concluded that the nature of the rate-limiting step is determined by the relative pK's of the nucleophile and group displaced.[9]

For anilide substrates it is suggested that $k_2 < k_{-1}$ so that

$$\text{rate} = k_2K(\text{RC}(\text{O})\text{X}\cdots\text{Enz}(\text{OH})) \quad (6)$$

where $K = k_1/k_{-1}$. In these reactions breakdown of a tetrahedral intermediate to form the acyl enzyme is rate limiting. Bruice and Schmir[10] have considered the implications of an identical scheme in a discussion of several hypothetical mechanisms for enzymatic acyl transfer. In these reactions substituent effects reflect a summation of effects of the rate step, k_2, and the equilibrium, K. The k_2 reaction can be further dissected into a protonation process which will be facilitated, and a C–N bond cleavage reaction which will be retarded by electron-donating substituents. The predominance of substituent effects on protonation is suggested by results obtained in studies of the hydronium ion catalyzed decomposition of carbamates in which it has been found that although the amine's leaving tendency decreases with increasing basicity, second-order rate constants for hydronium ion catalysis are proportional to the amine pK.[11] In contrast to the substituent effects on k_2, values of K, the equilibrium constant for formation of the tetrahedral intermediate, will be increased by electron withdrawal away from the acyl function by the aniline portion of the substrate. This will tend to make the net ρ for acylation less negative. If, however, $k_2 < k_{-1}$ *and* $k_1 \geq k_{-1}$, *i.e.*, the tetrahedral intermediate is formed in a preequilibrium reaction in which there is either no change or a negative change in free energy, the following rate law holds and substituent effects on

$$\text{rate} = \frac{k_2K}{K + 1}(\text{RC}(\text{O})\text{X}\cdots\text{Enz}) \quad (7)$$

K will have little effect on the rate. For example, if K equals 1 with the methoxyanilide, which contains an electron-*donating* substituent, the rate can only be doubled by substituent-mediated increases in K.

The assignment $k_{-1} > k_2$ in anilide reactions is supported by results obtained by Cunningham and Schmir in studies of the cyclization of 4-hydroxybutyranilide to butyrolactone.[12a] This reaction is considered to be an appropriate model for the chymotrypsin acylation since both reactions involve acyl transfer from aniline to an aliphatic alcohol. In the 4-hydroxybutyranilide reaction $k_{-1} = k_2$ at pH 7.1 and $k_{-1} > k_2$ at more alkaline pH's. The hydroxybutyranilide reaction has been interpreted in terms of a mechanism in which an anionic tetrahedral intermediate formed by attack of the neighboring hydroxyl group partitions between an uncatalyzed pathway, which gives C–O bond cleavage and regenerates the starting material, and a hydronium ion catalyzed pathway, which liberates aniline; the rates of these two reactions are equal at pH 7.1. Chymotrypsin acylation has been formulated in similar terms in Scheme I.[12b] In this scheme the general base catalyzed attack of the serine hydroxyl group gives an anionic tetrahedral intermediate along with the conjugate acid of imidazole. The anionic tetrahedral intermediate breaks down with C–O bond cleavage to regenerate the starting materials. This back reaction is identical with that proposed for the nonenzymatic analog except that from the principle of microscopic reversibility the imidazolium group must function as a general acid catalyst in the regeneration of starting materials. The breakdown patterns for the nonenzymatic and enzymatic reactions may be made identical if the imidazole

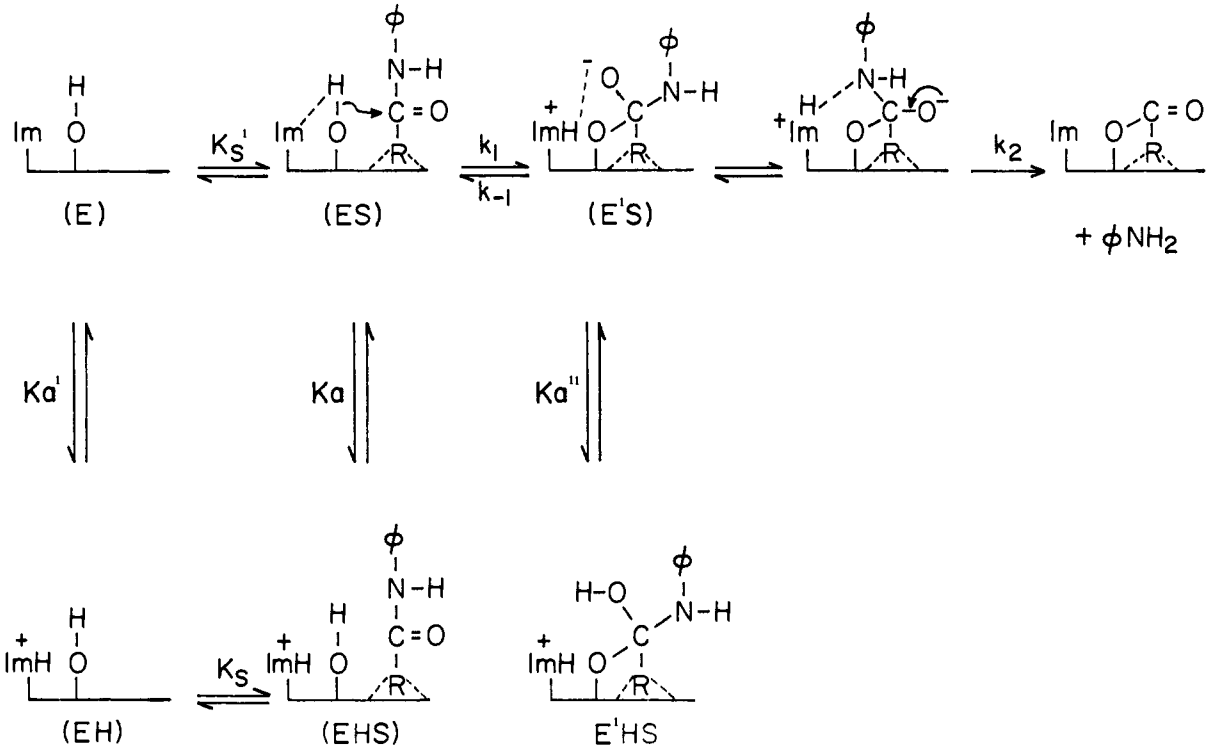
(9) W. P. Jencks and M. Gilchrist, *J. Am. Chem. Soc.*, 90, 2622 (1968).

(10) T. C. Bruice and G. L. Schmir, *ibid.*, 81, 4552 (1959).

(11) M. Caplow, *ibid.*, 90, 6795 (1968).

(12) (a) B. A. Cunningham and G. L. Schmir, *ibid.*, 89, 917 (1967). (b) A major inconsistency in the acyl-enzyme scheme has been observed in the kinetics of acetyltyrosinehydroxamic acid formation by chymotrypsin (M. Caplow and W. P. Jencks, *J. Biol. Chem.*, 239, 1640, 1964). The scheme given here includes an acyl enzyme but the results may also be accounted for in terms of a mechanism in which the tetrahedral intermediate is formed by attack of water on the anilide.

Scheme I



catalysis in the attack step is omitted so that the serine anion is the reactive species in the forward and reverse reactions. Alternatively, in the reaction in which imidazole functions as a general base catalyst for serine attack the proton may be very extensively removed so that in the reverse reaction the proton will only be slightly donated to the serine leaving group. In this case the back-reaction is essentially an uncatalyzed breakdown of the anion. Release of aniline from the anionic tetrahedral intermediate requires protonation of the leaving group by imidazolium. However, since imidazolium is a weaker acid than hydronium ion, unlike the hydroxyanilide reaction, the C–O cleavage reaction predominates under all conditions. Aniline protonation by imidazolium will be facilitated by electron donation so that ρ for the k_2 step will be negative.[13a]

It should be noted that for the case where $k_2 < k_{-1}$ it follows that kinetic studies provide no information concerning the mechanism of the attack step. The mechanism for formation of the tetrahedral intermediate given in Scheme I has been formulated so as to involve no change in charge in going from the first reaction to the rate-limiting transition state, which must have a net zero charge. While we consider this mechanism a likely one, other schemes for the attack step cannot be ruled out.

The assignment $k_1 > k_{-1}$, *i.e.*, $K > 1$, is required for interpretation of the results reported here in terms of Scheme I; *vide infra*. Although significant accumulation of a tetrahedral intermediate has been found to occur in several nonenzymatic reactions of nucleophiles with activated acyl compounds,[13b,c] this is not true for reactions of anilides. It is, therefore, necessary to propose that in the reaction of chymotrypsin with anilides the formation of a tetrahedral intermediate is made more exergonic either as a result of destabilization of the anilide or stabilization of the tetrahedral intermediate. Destabilization of the anilide may result from the enzyme binding the substrate in a form in which the amide resonance is inhibited so that the electrophilic character of the acyl group is enhanced. This may be considered to be a type of catalysis by strain, a process which has been reasonably well substantiated in several enzymatic and nonenzymatic reactions.[14] Ingles and Knowles[15] have concluded from an extensive analysis of the stereospecificity of chymotrypsin reactions that binding forces directed to the N-acylamino group are utilized for producing a high-energy form of the substrate.

Formation of the tetrahedral intermediate may also be favored if substrate binding forces are more favorable in the tetrahedral intermediate as compared with the Michaelis complex. This suggestion is supported by direct measurements of the free energy for formation of furoylchymotrypsin in which it has been found that this intermediate is several kilocalories more stable than a simple serine ester.[16] The change in free energy for

(13) (a) From the symmetry of acylation and deacylation the scheme outlined here must proceed in reverse in reactions of an acyl enzyme with an aniline. If the reactions of aniline and water are taken to be equivalent, a tetrahedral intermediate is required in acyl-enzyme hydrolysis if it is implicated in the acylation of the enzyme with anilides; (b) G. E. Lienhard, *J. Am. Chem. Soc.*, **88**, 5642 (1966); (c) D. R. Robinson, *Tetrahedron Letters*, 5007 (1968).

(14) (a) W. P. Jencks in "Current Aspects of Biochemical Energetics," N. O. Kaplan and E. P. Kennedy, Ed., Academic Press, New York, N. Y., 1966, p 273; (b) P. D. Boyer, *Proc. Intern. Congr. Biochem., 5th Moscow 1961*, **4**, 18 (1963); (c) R. Lumry, *Enzymes*, 1, 157 (1959).

(15) D. W. Ingles and J. R. Knowles, *Biochem. J.*, 108, 561 (1968).

(16) (a) P. W. Inward and W. P. Jencks, *J. Biol. Chem.*, **240**, 1986 (1965). The equilibrium constant measured here is for formation of an acyl enzyme from unbound ester and enzyme rather than from an enzyme–substrate complex so that there is no change in molecularity in the reaction. The correct values for the free energy for hydrolysis at pH 7 of furoylchymotrypsin and a furoyl serine ester are −6070 and −8820 cal/mol, respectively. The difference, which may be attributed to stabilization by binding to the enzyme, is 2750 cal/mol; this is the same as reported earlier (personal communication from W. P. Jencks); (b) R. M. Epand and I. B. Wilson, *ibid.*, **239**, 4145 (1965), have calculated an equilibrium constant for acyl-enzyme formation from a

conversion of a Michaelis complex composed of chymotrypsin and acetyltryptophan (neutral) to acetyltryptophanylchymotrypsin is −620–1150 cal/mol at 25°.[17] This is to be compared with a calculated free energy change of +3520 cal/mol (25°) for formation of an ester from acetic acid and N-acetylserinamide.[16a] Since the acyl enzyme is a serine ester it may be concluded that the acyl enzyme is significantly stabilized as compared with the Michaelis complex. Although a comparison has been made between the free energy change for acyl-enzyme formation and lactone synthesis[17a] the latter process is a poor model for the enzymatic reaction since the serine hydroxyl is significantly more acidic than an ordinary aliphatic alcohol[18] and the change in free energy for ester formation is linearly related to the alcohol pK.[19] Furthermore, lactone formation is undoubtedly driven toward completion, as compared with intermolecular esterification, by entropy changes associated with having a single molecule (hydroxy acid) give two products (lactone and water). In the enzyme reaction it is likely that the hydroxyl group of the carboxylic acid occupies the leaving group site both before and after acyl-enzyme formation so that the reaction is, in effect, unimolecular in both directions. Although the foregoing discussion has been concerned with the stability of the acyl enzyme it is likely that factors contributing to the stability of this species will have become manifest, at least in part, in the course of the formation of the tetrahedral intermediate.

Finally, the reasonableness of the proposal that tetrahedral intermediate formation is more energetically favorable in an enzymatic as compared with the corresponding nonenzymatic reaction should be considered in the light of the fact that at neutral pH the maximum rate of acylation of chymotrypsin with an acetyltryptophan anilide is more than 10^7 times as fast as intramolecular acyl transfer in 4-hydroxybutyranilide.[12a] It is not unlikely that several unusual catalytic processes are involved in this remarkable rate acceleration.

Results obtained with anilide substrates are consistent with a mechanism in which a substituent-dependent equilibrium reaction is interposed between the conversion of the Michaelis complex to the acyl enzyme. This conclusion is supported by the substituent dependence of K_m, which for acetyltyrosine compounds progressively decreases as the aniline substituent is made more electron withdrawing with K_m equal to 33.0×10^{-3} M with the p-methoxyanilide and 1.28×10^{-3} M for the p-chloroanilide.[5e] It is unlikely that these variations are related to substituent effects on the simple adsorbtion process since the binding of substrates to chymotrypsin is insensitive to the nature of the group bound to the susceptible acyl linkage. For example, the binding constants determined by equilibrium dialysis for acetyl-D-tryptophan, which is anionic at the pH's studied, and acetyl-D-tryptophan amide are approximately equal.[20]

Also, in reactions of amines and alcohols with furoylchymotrypsin, which is the reverse of the acylation reaction with an amide and ester substrate, respectively, relatively little specificity is observed for the nucleophilic acceptor except for a general trend in which there is a progressive increase in reactivity as the hydrocarbon nature of the nucleophile increases.[16a] If the reaction of nucleophiles occurs from the leaving group site, which is required by microscopic reversibility, these results suggest that the leaving group site does not possess the requisite specificity to account for the large changes in K_m observed with the relatively small changes in the structure of the anilides. Finally, this possibility may be rejected on the basis of results reported here in which it has been found that K_m's for acetyltryptophan p-chloro- and p-methoxyanilides become equal at pH 5.8.

Application of Scheme I. Under conditions where (S) > (E) the rate law derived by assuming a steady state for all forms of the enzyme through E'S is

$$\frac{\text{rate of acylation}}{\text{total enzyme}} = \frac{k_2 K K_a K_a' K_a''(\mathrm{S})}{K_S' K_a K_a' K_a'' + K_S' K_a K_a''(\mathrm{H}) + K_a K_a' K_a''(\mathrm{S}) + K_a' K_a''(\mathrm{S})(\mathrm{H}) + K K_a K_a' K_a''(\mathrm{S}) + K K_a K_a'(\mathrm{H})(\mathrm{S})} \quad (8)$$

where the equilibrium constants are formulated in terms of dissociation and K = (E'S)/(ES). The apparent K_m derived from this scheme is[21]

$$K_m = \frac{\dfrac{K_S' K_a K_a''(K_a' + (\mathrm{H}))}{K_a'(K_a'' + K K_a)}}{\dfrac{K_a K_a''(1 + K)}{K_a'' + K K_a} + (\mathrm{H})} \quad (9)$$

which at high pH[22] reduces to

$$K_m = K_S'/(1 + K) \quad (10)$$

K_m is, therefore, inversely proportional to K at pH's where the enzyme is fully ionized (pH 8 and above).

From eq 9, for the case where $K < 1$, at high pH

$$K_m = K_S' \quad (11)$$

and at low pH

$$K_m = K_S' K_a/K_a' \quad (12)$$

Since K is proportional to electron withdrawal it is likely that this constant will be small with the amide substrate. That this is the case for acetyltryptophan amide is indicated by the fact that the kinetically determined K_m for this substrate has been demonstrated to be a binding constant.[24] Furthermore, since K_m for acetyltryptophan amide is invariant in the pH range 8.1–6.0[23] it may be concluded that $K_a = K_a'$.

It is proposed that $K \geq 1$ with anilide substrates. From eq 9, at low pH

Michaelis complex composed of chymotrypsin and acetyl-L-tyrosine-hydroxamic acid which is about 200 times greater than the constant for reaction of a hydroxamic acid with an alcohol. In this calculation it is assumed that noncovalent interactions between the acyl residue and the enzyme are the same in the Michaelis complex and the acyl enzyme.

(17) (a) F. J. Kezdy and M. L. Bender, *J. Am. Chem. Soc.*, **86**, 938 (1964); (b) F. J. Kezdy, G. E. Clement, and M. L. Bender, *ibid.*, **86**, 3690 (1964).

(18) T. C. Bruice, T. H. Fife, J. J. Bruno, and N. E. Brandon, *Biochemistry*, 1, 7 (1962).

(19) J. Gerstein and W. P. Jencks, *J. Am. Chem. Soc.*, **86**, 4655 (1964).

(20) C. H. Johnson and J. R. Knowles, *Biochem. J.*, **101**, 56 (1966).

(21) The apparent K_m, which will be referred to as K_m, is equal to the substrate concentration necessary for half maximal velocity.

(22) A group with a pK of 8.6 is involved in chymotrypsin binding.[23] The results reported here were obtained at pH 8.1 and below and the ionization of this group may, therefore, be neglected.

(23) A. Himoe, P. C. Parks, and G. P. Hess, *J. Biol. Chem.*, **242**, 919 (1967).

(24) K. G. Brandt and G. P. Hess, *Biochem. Biophys. Res. Commun.*, **22**, 447 (1966).

$$K_m = K_S' \frac{K_a K_a''}{K_a'(K_a'' + KK_a)} \quad (13)$$

K_m is, therefore, pH dependent (compare eq 10 and 13) unless $K_a = K_a' = K_a''$, in which case eq 10 holds at high and low pH. If the assignment $K_a = K_a'$ indicated for the amide holds for all substrates

$$K_m = K_S' \frac{K_a''}{K_a'' + KK_a} \quad (14)$$

at low pH. Accordingly, K_m is equal to K_S' at low pH if $K_a'' > KK_a$[25] and K_m will, therefore, increase with decreasing pH. For the reverse case in which $K_a'' < KK_a$

$$K_m = K_S' \frac{K_a''}{KK_a} \quad (15)$$

and K_m will either increase or decrease at more acidic pH's depending on relative size of the various constants in eq 15. Irrespective of whether K_m is made larger or smaller with decreasing pH, values for K_m will follow a titration curve of a hypothetical ionizing group with an apparent K_a which is defined as

$$K_a(\text{apparent}) = \frac{K_a K_a''(1 + K)}{K_a'' + KK_a} \quad (16)$$

Inspection of Scheme I suggests that as a result of the relatively stable hydrogen bond in the conjugate base of E'HS this form of the enzyme will be more acidic than either EH or EHS. The assignment $K_a'' > K_a K$ is, therefore, a likely one. That this is the case is supported by results obtained with acetyltryptophan and acetyltyrosine[5e] *p*-chloroanilides which show a progressive increase in K_m with decreasing pH. On the other hand, the K_m for acetyltryptophan *p*-methoxyanilide shows relatively little change in the pH range 8.0–5.8. This may be taken to indicate that the K_m obtained for this compound at high pH is relatively unaffected by the denominator term in eq 10. The large change observed for the chloroanilides suggests that K is large for these compounds, as would be expected.

K_m for the acetyltryptophan anilides and the simple amide are approximately equal at pH 5.8. The scatter in the results obtained at lower pH's made it impossible to determine whether K_m is subject to further pH effects. It is believed that values for K_m reported here for anilides are not binding constants, irrespective of pH, since the dimethyl sulfoxide used in these studies could be expected to act as a competitive inhibitor to the enzyme.[26]

From eq 8

$$V_{max} = \frac{\frac{k_2 K K_a K_a''}{K_a'' + KK_a}}{\frac{K_a K_a''(1 + K)}{KK_a + K_a''} + (H)} \quad (17)$$

This reduces to

$$V_{max} = k_2 K/(1 + K) \quad (18)$$

at high pH. V_{max} is, therefore, independent of K for substrates for which $K \gtrsim 1$, as described above. Values of V_{max} are pH dependent and will follow a titration curve the midpoint of which is an apparent K_a identical with that given in eq 16 for K_m. From eq 16, if $K_a'' > KK_a$, *i.e.*, if E'SH is a relatively strong acid

$$K_a(\text{apparent}) = K_a(1 + K) \quad (19)$$

so that the apparent K_a will depend on the nature of the substrate and will increase for substrates for which K is large. In accord with this hypothesis the apparent pK's for the maximum rate of hydrolysis of acetyltryptophan *p*-methoxy- and *p*-chloroanilides are approximately 6.6 and <6.0, respectively.

Other mechanisms in which the importance of acid catalysis is stressed have been suggested to account for the negative ρ for acylation of chymotrypsin with anilides. Parker and Wang[5e] have recently proposed a mechanism involving a combination of specific base–general acid catalysis for these reactions. This mechanism, in which a serine anion attacks a protonated substrate, has been convincingly ruled out for the acylation of chymotrypsin with acetyltyrosine ethyl ester.[27] To avoid the complications associated with proposing a separate mechanism for each type of substrate we are inclined to reject this mechanism for anilides. A simple specific base–general acid mechanism has been ruled out for reactions of amines and alcohols with furoylchymotrypsin.[16a] These reactions are the reverse of the acylation reaction with amides and esters so that for the specific base–general acid mechanism to hold it is necessary that the reaction proceeds *via* a different path in the forward and reverse direction. In addition to these points, previously proposed mechanisms do not account for the following observations. (1) K_m for anilides is decreased by electron-withdrawing substituents. (2) K_m is severely pH dependent in the neutral pH range with substrates containing electron-withdrawing substituents. (3) The apparent pK for acylation with anilides is dependent on the nature of the substrate. (4) ρ for acylation of chymotrypsin with esters is positive.

The results reported here are consistent with catalytic schemes other than the one suggested in Scheme I. For example, the equilibrium reaction (K) in Scheme I may be omitted and it may be assumed that the acid dissociation constant for the Michaelis complex (K_a) depends on the nature of the substrate, so that in some cases $K_a \neq K_a'$. With substrates for which $K_m \neq K_a'$, K_m will be pH dependent (see eq 11 and 12) and the apparent K_a for activity will depend on the nature of the substrate. For this mechanism to hold additional factors must be proposed to account for the substituent dependence of V_{max}. Aside from the incompleteness of this mechanism we are disinclined to accept this scheme because of difficulties in envisioning a simple mechanism for a substrate-induced significant perturbation in the enzyme pK.

(25) In mechanism II of ref 10, which is similar to Scheme I given here, the acid–base properties of the enzyme–tetrahedral intermediate complex are not considered. The relationships derived from mechanism II hold only if this intermediate is weakly basic, *i.e.*, if $K_a'' > KK_a$.

(26) (a) J. L. Miles, E. Morey, F. Crain, S. Gross, J. San Julian, and W. J. Canady, *J. Biol. Chem.*, **237**, 1319 (1962); (b) J. L. Miles, D. A. Robinson, and W. J. Canady, *ibid.*, **238**, 1318 (1963).

(27) M. L. Bender and F. J. Kezdy, *J. Am. Chem. Soc.*, **86**, 3704 (1964).